Evidence for Dimorphism in Rabbit Hemoglobins* (33147)

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Multiplicity of hemoglobin molecules in a single species and molecular differences among species have been matters of great interest in molecular biology. After the pioneer work of Pauling and subsequent studies on the mechanism of sickle cell anemia, numerous investigations have been carried out on human hemoglobin mainly in connection with hereditary diseases. As a result, a number of different hemoglobins have been identified (1). Such an intraspecies multiplicity of hemoglobin has also been found in many species of mammals and other animals (2–4).

In the course of studies on induced hemoglobin biosynthesis (5–6), the authors observed the presence of two types in peptide maps of chymotryptic digests of hemoglobin from individual rabbits. This report presents evidence for dimorphism of rabbit hemoglobin, which is probably due to differences in amino acid content of the β-chain of isolated hemoglobins.

Materials and Methods. Twenty-four rabbits of New Zealand and Dutch strains, of both sexes and different ages, were used. Hemoglobin was prepared and purified according to Drabkin's method (7). Type A hemoglobin and type B hemoglobin were obtained from the blood of individual rabbits after differences in peptide patterns among

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individual hemoglobins had been observed. The blood was collected in a tube containing 3.2% sodium citrate solution, and washed 5–6 times with saline to remove the serum and white blood cells. The erythrocytes thus collected were hemolyzed by the addition of 0.1% saponin solution, and the stroma was removed by ultracentrifugation. The supernatant containing hemoglobin was dialyzed against 2.8 *M* potassium phosphate buffer (pH 6.8) at 4°C. Hemoglobin was crystallized by the addition of ice-chilled, saturated ammonium sulfate solution. The crystals collected were dissolved and dialyzed against distilled water at 4°C, and finally lyophilized.

The preparation of globin from hemoglobin was according to the procedure of Rossi Fanelli *et al.* (8). Acetone, containing 0.3% (w/w) of 2 *N* hydrochloric acid, was prechilled to —20°C and added to the ice-chilled hemoglobin solution with stirring. A yellowish-white, flocculent precipitate was collected and dissolved in distilled water. The solution was dialyzed against distilled water and then lyophilized, after the removal of a precipitate which appeared during dialysis.

Chernoff's chromatographic technique (9) was applied for the separation of subunits from globin. Globin was dissolved in 11.7% formic acid, the solution was applied to an Amberlite IRC 50 ion-exchange resin column, and the elution was accomplished with an interrupted urea gradient from 2 *M* to 8 *M* at pH 1.9. The α-chain was eluted first at a concentration of 5.3 *M* urea and the β-chain was eluted at a concentration of 6.6 *M*. A third fraction with a slightly yellowish color was eluted with higher urea concentration, and found to yield the same peptide map as the β-chain, but with less α-chain contamination. Therefore, subsequent digests were carried out using this fraction.

Hemoglobin, globins, and subunits thus obtained were subjected to chymotryptic digestion, in most cases, at pH 8.3–8.4 for 20 hours at 37°C. Trypsin was also used for digestion under the same conditions. (Both enzymes were purchased from Worthington Biochemical Corporation.)

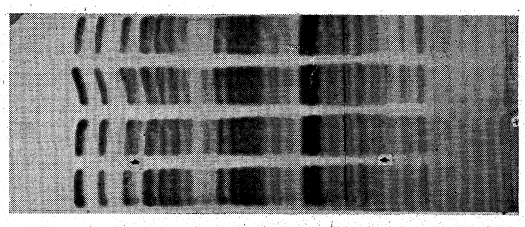

FIG. 1. Paper electropherogram of chymotryptic digests of rabbit hemoglobins. Arrows indicate additional peptide bands in center patterns. Pyridine acetic acid buffer, pH 6.4, 41 V/cm, 2 hours.

The peptide maps of these digests were obtained as follows. The digested materials were lyophilized, dissolved in pyridine–acetic acid buffer (pH 6.4) and applied to Whatman 3MM paper for high voltage electrophoresis in the horizontal plane (41 V/cm; 2 hours; pyridine–acetic acid buffer pH 6.4). After electrophoresis, the paper was air-dried and subjected to ascending chromatography, if a two-dimensional peptide map was desired. The solvent system consisted of *n*-butanol–acetic acid–water (3:1:1) and chromatography was carried out for 6 hours. Two mg of digest was applied as a spot for peptide mapping, and 2 mg/inch was applied as a streak for single dimension high voltage electrophoresis. A 0.2% solution of ninhydrin in acetone was used to visualize the peptide map.

Amino acid analysis of the subunits was performed with the use of an amino acid analyzer, after hydrolysis with 6 *N* hydrochloric acid for 24, 48, and 72 hours. Residue numbers were determined by averaging molar ratio values derived from 8 different amino acids taken as unity. Amino acid anaylsis of several peptides obtained by preparative electrophoresis was also carried out.

Results and Discussion. Figure 1 is a paper electropherogram of the chymotryptic digests of hemoglobins from individual rabbits. The arrows indicate at least two additional peptide bands occurring in the digests derived from the two hemoglobins represented by the center patterns. The presence of the two hemoglobin types, i.e., the type without (Type A) and with (Type B) these extra bands, was confirmed in the two-dimensional peptide maps as shown in Fig. 2. The peptide

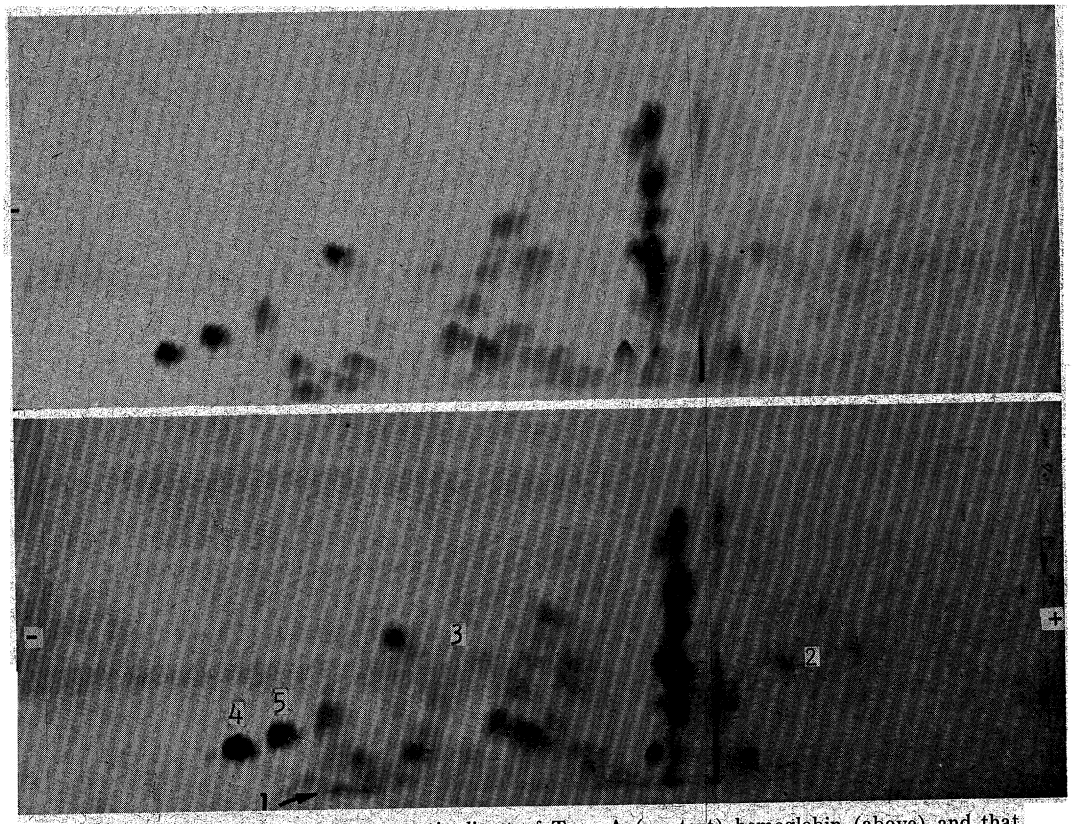

FIG. 2. Peptide maps of chymotryptic digest of Type A (see text) hemoglobin (above) and that of Type B hemoglobin (below). Electrophoresis; pyridine acetic acid buffer pH 6.4, V/cm, 2 hours. Ascending chromatography; *n*-butanol–acetic acid–water (3:1:1).

map (below) of the chrymotryptic digest of Type B hemoglobin clearly revealed two extra spots marked as 1 and 2, as compared with the map (above) of Type A hemoglobin. The appearance of the two types was reproducible with respect to individual animals, i.e., hemoglobin from animals examined monthly for more than a year yielded the same type of peptide map pattern. No relation was noted between the type and strain, sex or age of the animals. Repeated injections of phenylhydrazine to induce reticulocytosis had no effect on the type.

When hemoglobin preparations were not completely homogenous, it was found that additional spots appeared in the region of the peptide map marked 3 in Fig. 2. This was usually the case for hemoglobin obtained from the mother liquors after initial crystallization. When digests of hemoglobins, shown to be completely homogenous by disc gel electrophoresis, were examined, they showed only the differences indicated in Figs. 1 and 2, and clearly demonstrate a multiplicity of hemoglobin in the rabbit.

Peptide maps were also made for the chymotryptic digests of globins prepared from the typed hemoglobins. The types of peptide map, one without and one with the extra spots, were also observed for globin digests, each type corresponding to the type of original hemoglobin. This fact suggests the difference in the peptide map between Type A hemoglobin and Type B is inherent to the protein globin.

Though the peptide map of the globin digest was almost identical with that of the hemoglobin digest, two spots which are shown to travel very rapidly toward the cathode, 4 and 5 in Fig. 2, virtually disappeared in the case

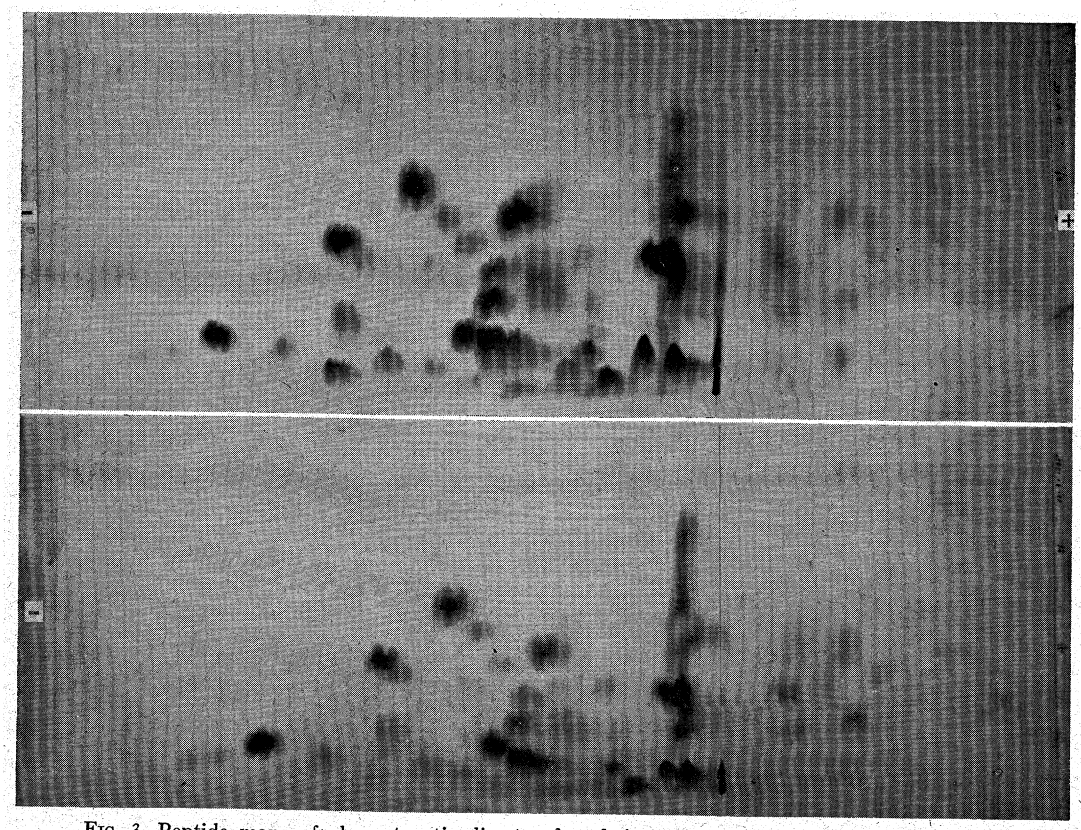

FIG. 3. Peptide maps of chymotryptic digests of α-chains of Type A hemoglobin (above) and that of Type B (below) (conditions as in Fig. 2).

of the globin digests. Amino acid analyses of those peptides revealed the amino acid content as lysine, glycine, and serine for peptide 4 and as arginine only for peptide 5. Presumably these two peptides are located in the regions which are responsible for the binding of heme and globin moieties.

In order to determine which chain of globin is responsible for the difference between the two types, peptide maps were made for the chymotryptic digests of both α- and β-chains derived from each type of globin. As shown in Fig. 3, α-chain digests from both types of globins gave essentially identical peptide maps, while the difference was observed in the maps of the two β-chain digests as shown in Fig. 4. It was concluded that a difference in β-chain composition and /or structure is responsible for the apparent multiplicity of rabbit hemoglobin.

To support this finding, amino acid analyses were carried out for each subunit. As shown in Table I, an essentially identical amino acid composition was found for each α-chain from the two types of hemoglobin, with the content of no amino acid differing between types by more than 0.5 residue, except for leucine with a difference of 0.6 residue. All other differences are 0.5 of a residue or less. This excess leucine in the α-chain of Type A hemoglobin does not appear to be compensated for by a decrease in content of any other amino acid, and probably results from an inexplicable error in the analyses. On the other hand, there appear to be significant differences in valine and lysine content of the β-chain of the two types of hemoglobin.

The β-chain from Type A hemoglobin seemed to have one more lysine residue and one less valine residue as compared with that from Type B hemoglobin. The results of the

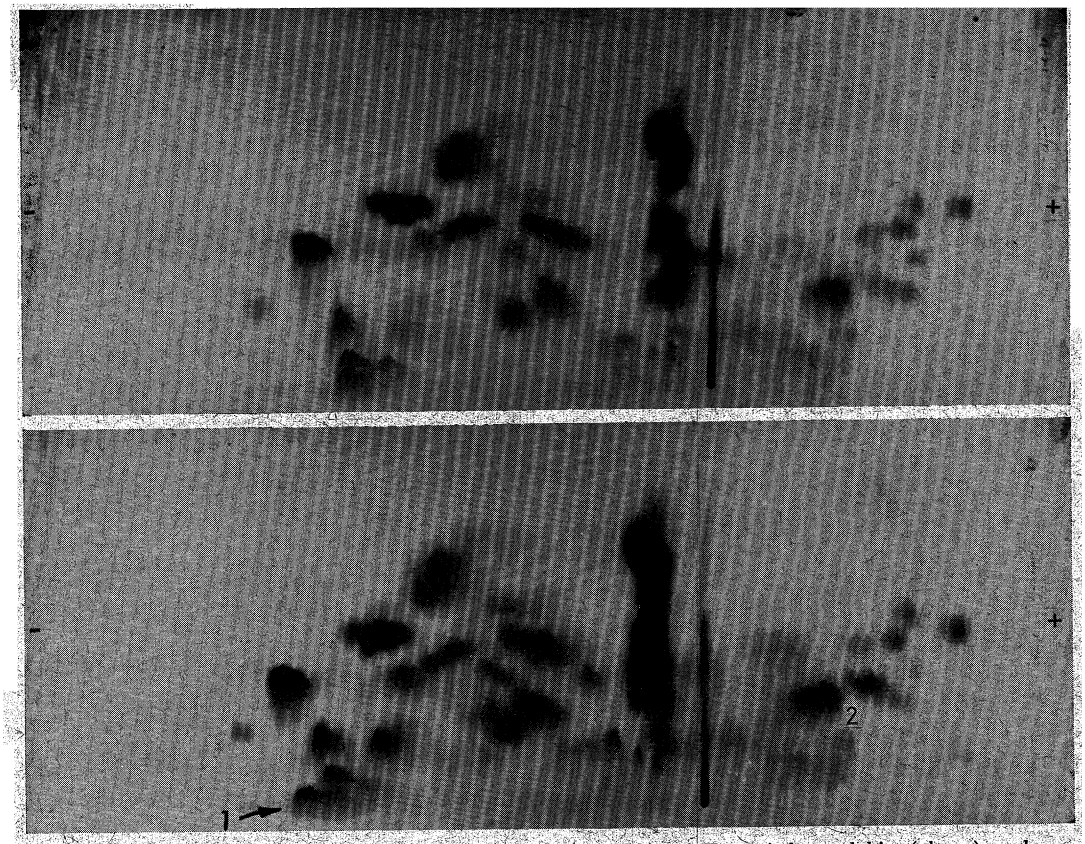

FIG. 4. Peptide maps of chymotryptic digests of β-chains of Type A hemoglobin (above) and that of Type B (below) (conditions as in Fig. 2).

amino acid analyses indicating differences in the β-chains and not in the α-chains, agree with the conclusion deduced from the peptide maps.

The amino acid composition of the subunits of rabbit hemoglobin obtained here coincides fairly well with that reported by Diamond and Braunitzer (10), by Naughton and Dintzis (11) and by Von Ehrenstein (12) though there are some discrepancies in overall amino acid composition, but of a minor nature.

Amino acid analyses were also obtained from the peptides indicated as 1 and 2 in Fig. 2. Peptide 1 was found to be composed of 1 glycine, 1 aspartic acid, 1 serine, 1 proline, 1 alanine, 1 valine, 2 lysines, and 1 histidine. This composition coincides with the amino acid sequence from No. 56 and No. 63 in the β-chain of human and horse hemoglobins, with the exception of the presence of serine. The amino acid composition of peptide 2 was 1 aspartic acid, 3 or 4 serines, 1 glutamic acid, 1 or 2 glycines, 2 alanines, 1 valine, 1 methionine, 1 leucine, 1 phenylalanine, and 1 histidine. The composition seems to be similar to the amino acid sequence from No. 43 to No. 55 in the β-chain of the above two hemoglobins. Hence, the difference(s) between Type A and B rabbit hemoglobins may be located in that region of the β-chain. Tryptic digests of Type A and B β-chains also revealed differences in peptide maps obtained in the same way as for the chymotryptic digests.

The data presented above have demonstrated the multiplicity of rabbit hemoglobin. Long term observation has established the consistency of hemoglobin type in each of the rabbits used. Apparently the present data

TABLE I. Amino Acid Composition of the Subunits of Both Types of Rabbit Hemoglobin.[a]

	α-chain		β-chain	
Amino acid	Type A	Type B	Type A	Type B
Asp	11.0	11.3	12.5	12.2
Thr	11.2	11.4	4.8	5.2
Ser	11.4	10.9	10.3	10.4
Glu	8.2	8.2	13.9	13.6
Pro	7.0	7.2	4.7	4.8
Gly	9.2	9.3	11.3	11.6
Ala	12.9	13.1	15.1	15.2
Cys (half)	0.6	0.6	0.7	0.7
Val	9.4	9.7	16.8	17.6
Met	1.0	0.9	1.0	0.9
Ileu	3.0	2.9	1.2	0.9
Leu	18.0	17.4	18.2	18.5
Trp	2.6	2.7	2.6	2.8
Phe	7.2	7.5	7.9	8.2
Lys	12.0	12.1	12.6	11.9
His	10.1	10.5	9.3	9.5
Arg	3.0	3.0	3.2	3.3

[a] Values are residue numbers calculated on basis of molar ratios.

refute the observation based on results of agar and paper electrophoreses (13–15) (single type of rabbit hemoglobin), but are in line with the finding obtained from ^{59}Fe incorporation (16) and other procedures (12, 17) (multiplicity of rabbit hemoglobins).

For the 24 rabbits used in the present experiments, the frequency of the occurrence of Type A hemoglobin was 0.33. The number of animals examined is too small to establish an acceptable frequency value for the distribution of the two types of hemoglobin. It is hoped, however, that future breeding experiments among rabbits with different hemoglobins will yield information to ascertain the genetic aspect of this dimorphism.

It is of interest that the difference between the two types was found in the β-chain, since differences found in hemoglobins of other animal species were confined mainly to the β-chain (4). If the multiplicity of rabbit hemoglobins is under genetic control as are other hemoglobins of other species, it would appear on the basis of the genetic code (18) that a substitution of lysine for valine would require at least two mutational events, assuming that lysine and valine occur at the same position in the β-chain of the two types of hemoglobin. It could be theorized that other hemoglobins exist, as yet undiscovered, which contain glutamic acid, isoleucine, and methionine substitutes at the position, having passed through an intermediate mutation in going from lysine to valine, or vice versa. It is also conceivable that such mutations had little or no survival value and will not be found to exist in nature.

Summary. The hemoglobin of 24 rabbits was analyzed by the "finger-printing" technique and amino acid analyses. The data revealed the presence of dimorphism in rabbit hemoglobin. The difference in composition and/or structure appears to be located in the β-chain.

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